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Clonal Structure and Genetic Differentiation in the Serpentine Plant *Japonolirion osense* (Petrosaviaceae) Using AFLP Markers

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Japonolirion osense Nakai (Petrosaviaceae) is a vulnerable plant of serpentine soils known to occur only on Mt. Shibutsu and Mt. Tanigawa (Gunma Prefecture), and at Toikanbetsu (Hokkaido), Japan. We investigated the clonal structure and genetic differentiation in these populations using amplified fragment length polymorphism (AFLP) analysis. *Japonolirion osense* reproduces via rhizomes. The AFLP phenotype patterns indicated that genets were about 1-5 m in diameter. Some clones of the plant from the Mt. Shibutsu population had invaded open habitat to about 15 m where erosion from snow had caused landslides. Our population study showed that the populations of *J. osense* on Hokkaido and Honshu are genetically different.

Key words: AFLP, clonal growth, *Japonolirion osense*, serpentine plants, population differentiation

Serpentine floras are distinct and often contain many globally rare and endangered endemic species (Stevanović *et al.* 2003). Several studies conducted in major serpentine areas in the Balkans and Scandinavia have discussed the relationships between distinct endemic floras and the chemical and physical influence of serpentine soils, which contain heavy metal ions such as Ni and Co (Proctor & Woodell 1975, Johnston & Proctor 1981, Westerbergh & Sauna 1992, Robinson *et al.* 1997, Mengoni *et al.* 2003, Berglund *et al.* 2004). Serpentine soils can be dry due to a lack of organic materials (Brooks 1987). Physical factors, such as substrate setting and elevation, are thought to be important in controlling the distribution of plant species in a serpentine area in Italy (Chiarucci *et al.* 2001). In these chemically and physically stressful environments, some plants have developed traits that enable them to tolerate these factors. Additionally, plants that occur in serpentine soils are relatively free from competition, since the edaphic conditions are unfavorable for other plants (Kitamura 1950, Proctor & Woodell 1975).

Japonolirion osense Nakai, a perennial herb endemic to Japan, is a plant of sandy, wet, serpentine soils (Kitamura 1950). It is known from three populations disjunctly distributed 20 to 900 km apart on Mt. Shibutsu and Mt. Tanigawa in Gunma Prefecture, Honshu, and at Toikanbetsu in northwestern Hokkaido (Nakai 1930, Tatewaki 1931, Hara & Kanai 1959, Fig. 1). A number of other endemic and relict species grow with *Japonolirion*.

Recent molecular phylogenetic analysis supports the taxonomic placement of *Japonolirion* in the Petrosaviaceae rather than in the Liliaceae (Fuse & Tamura 2000). *Japonolirion osense* has a somatic chromosome number of $2n = 24$ (Sato 1942). Whether it is self-compatible or self-incompatible is

TABLE 1. Locations of *Japonolirion osense*. N = number of collections; number in parenthesis corresponds to number of phenotypes detected from AFLP.

location	subpopulation	latitude	longitude	altitude (m)	N
Shibutsu	shi1	36°53'41" N	139°10'19" E	2130	37 (8)
	shi2	36°54'09" N	139°10'21" E	2220	17 (9)
	shi3	36°54'10" N	139°10'24" E	2214	40 (25)
	shi4	36°54'19" N	139°10'46" E	2028	17 (5)
	shi5	36°53'31" N	139°10'22" E	2055	19 (5)
Tanigawa	tani1	36°49'54" N	138°55'58" E	1822	20 (13)
Toikanbetsu	te1	44°58'26" N	142°07'51" E	81	20 (17)
	te2	44°58'24" N	142°08'08" E	113	12 (7)
	te3	44°58'27" N	142°07'47" E	75	36 (12)

unknown. Plants from Gunma Prefecture and Hokkaido were once considered two different species (Tatewaki 1931). The plant found in Gunma Prefecture by Hara & Nakai (1930) were given the Japanese name *ozesou*, while those from Hokkaido were named *teshiosou* by Makino & Tatewaki (1931). The population in Hokkaido has since been treated as a variety of the plants from Gunma Prefecture.

Japonolirion osense propagates via rhizomes, and it has been suggested that it has survived because of its reproductive system; clonal growth via underground rhizomes allows it to overcome disturbances caused by landslides (Tsukui & Takahashi 1995). Some rhizomes have survived for at least 24 years (Tsukui & Takahashi 1995). Tomimatsu *et al.* (2004) used allozyme analysis to evaluate the genetic diversity of *J. osense* and reported that populations in Toikanbetsu in the Teshio region of northern Hokkaido showed moderate genetic diversity compared to other clonal plants.

Many plants that grow in serpentine, including *Japonolirion osense*, are rare endemics because of their restricted habitat. Therefore, knowledge of their biology is needed so that they may be managed scientifically.

In this study, we determined (1) the clonal structure within populations and (2) the genetic variation within and among populations of *Japonolirion osense* at three locations using the amplified fragment length polymorphism (AFLP) technique (Vos *et al.* 1995) to identify genets and to detect genetic variation and genetic divergence among populations. This method has been used successfully in many plant species due to its high reproducibility, large number of assayed loci, and genome-wide distribution of markers (Powell *et al.* 1996). This method is particularly well-suited for investigations at the intraspecific level and the identification of clonal structure (Suyama *et al.* 2000).

Materials and Methods

Study sites and field methods

Our study was conducted on Mt. Shibutsu and Mt. Tanigawa in Gunma Prefecture, and on Mt. Teshio, in Toikanbetsu, Hokkaido, Japan (Fig. 1). The vegetation on Shibutsu and Tanigawa is snow-patch vegetation that consists mainly of alpine plants, such as *Sieversia pentapetala* Greene and *Anemone narcissiflora* var. *nipponica* Tamura. In Gunma Prefecture, the habitat of *Japonolirion osense* ranges from 1800 to 2200 m in elevation, whereas at Teshio, it grows at low elevations (80-100 m) on wet, exposed riverside slopes where the surrounding area is covered with *Picea glehnii* Masters. At each location, the habitat is susceptible to landslide disturbances because of the unstable substrate and snow erosion. The substrate at Shibutsu consists of serpentine and rhyolite, which, like serpentine, is unstable and can result in landslides. This habitat is conspicuous because of the influence of snow erosion and need for vegetation restoration.

We sampled several subpopulations of *Japono-*

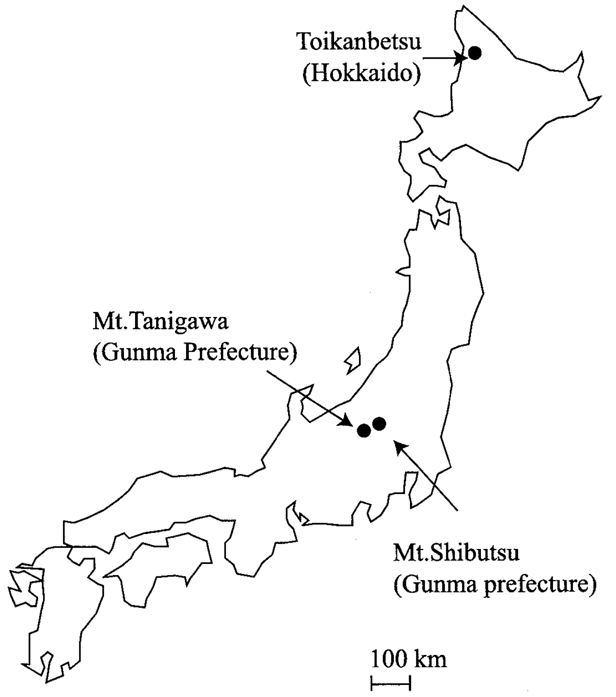

FIG. 1. Present distribution of *Japonolirion osense*.

lirion osense at each location, as shown in Table 1. We collected leaves from individuals in five subpopulations at Shibutsu, one population at Tanigawa, and three subpopulations at Teshio in July and August 2003 and 2004. Samples were collected at 1 m intervals in a patch more than 1 m long; one sample was from a patch of less than 1m long. To minimize the impact of sampling on wild populations, we collected young leaves (3 cm long), which were sufficient to extract genomic DNA, and dried them using silica gel.

Number of flowers per scape and size of flowering scape

Plants of *Japonolirion osense* differed in size between Gunma Prefecture and Hokkaido due to differences in soil nutrients and elevation at the different locations. Therefore, to quantify morphological differences between populations, we measured the scape and counted the number of flowers per scape at Teshio and Shibutsu. The results were compared using the Welch Test (unequal variance Student's t-test) with SPSS 11.0 software.

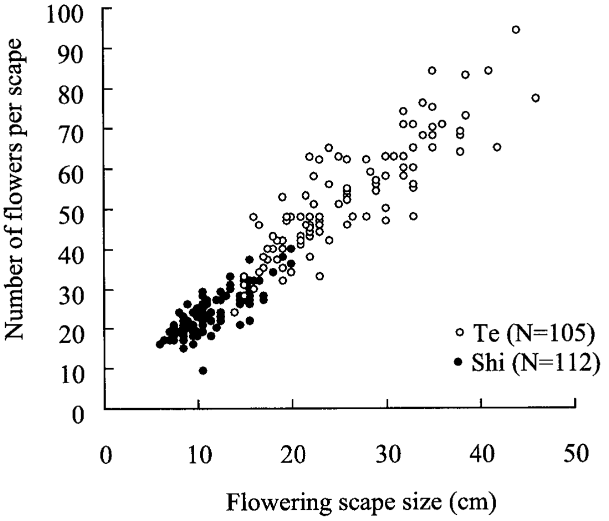

FIG. 2. Length of flowering scape and number of flowers per scape. Open and closed circles indicate data from Teshio and Shibutsu. (N = number analyzed)

DNA extraction

Total genomic DNA was extracted from 20-50 mg of leaves dried in silica gel using a DNA extraction kit (Plant Genomic DNA Extraction Miniprep System; Viogene, Sunnyvale, CA) according to the manufacturer's protocol. The extracted DNA was diluted in 100 μl of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and stored at -20°C.

AFLP analysis

The extracted DNA was digested with restriction enzymes (*Mse*I and *Eco*RI; New England Biolabs, Beverly, MA), ligated (T4DNA-Ligase, New England Biolabs) to adapters and preamplified using the AFLP Ligation and Preselective Amplification Module for regular genomes following the manufacturer's instructions (Applied Biosystems, Foster City, CA). The restriction-ligation reactions were incubated for 9 h at 25°C on a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) before performing the polymerase chain reaction (PCR) according to the recommended protocol. Deviating from the protocol, the restriction-ligation reactions were run in a reaction volume of 5.5 μL, and the preselective and selective PCRs were run in a reaction volume of 10 μL. Selective PCR

TABLE 2. Percentage of polymorphic fragments detected using three primer pairs in a total of 101 phenotypes.

Primer pair	1	2	3	Total
Eco RI	ACA	ACT	ACT	
Mse I	CTG	CAC	CTA	
No. of total fragments	71	57	81	209
No. of polymorphic fragments	53	40	41	134
No. of monomorphic fragments	18	17	40	75
Percentage of polymorphic fragments (%)	74.6	70.2	50.6	64.1
Estimated DNA-molecular weight (bp)	52-461	68-496	58-461	

was done using three primer combinations that gave clear, unambiguous scoreable results (*Eco*RI FAM-ACT-*Mse*I-CAC; *Eco*RI FAM-ACA-*Mse*I-CTG; EcoRI FAM-ACT-MseI-CTA). The fluorescence-labeled selective amplified products were electrophoresed with an internal size standard (Gene-Scan-500 [ROX], Applied Biosystems) using an ABI3100 sequencer (Applied Biosystems).

Data analysis

Fragments were manually scored for a reproducible range of fragment sizes for each primer combination using GeneScan 3.7 software (Applied Biosystems). Each fragment identified visually was treated as a separate putative locus, then scored as present (1) or absent (0). We constructed a 0/1 data matrix comprising all individuals and all polymorphic fragments. Based on these binary data, genetic parameters were calculated. To estimate differentiation among populations, the genetic distances among populations were calculated using the program GenAlEx v5 (Peakall & Smouse 2001). A neighbor-joining tree of the populations based on Nei's (1978) genetic distance was constructed using PAUP4.0 (Swofford 2001). For the analysis of molecular variance (AMOVA), genetic variation was partitioned at three levels: among locations, among subpopulations within locations, and within subpopulations. The components of the variance portioned within and between subpopulations were estimated from a Euclidean distance matrix using GenAlEx5 (Peakall & Smouse 2001). The significance of the AMOVA variance components was tested using permutation procedures.

Results

Plant size and number of flowers

The size of the flowering and number of flowers per scape were plotted on a graph (Fig. 2). The average parameters for the size of the flowering scape in plants from Teshio and Shibutsu were 25.75 ± 7.48 and 10.98 ± 3.09 (mean ± SD). The average parameters for the number of flowers per scape in Teshio and Shibutsu were 52.37 ± 14.09 and 23.39 ± 5.32. The average parameters were about twice as large for the plants from Teshio compared to those from Shibutsu, and those differences were significant ($p < 0.001$).

Genetic variation within and among subpopulations by AMOVA

A total of 218 samples, including ramets from three locations, were collected and analyzed using AFLP markers. With the three primer combinations used, 209 fragments between 52 and 496 bp each were observed (Table 2). Seventy-five fragments (35.9%) in the total dataset were monomorphic and 134 (64.1%) fragments were polymorphic (Shibutsu, Tanigawa, and Teshio). As a result, we found 36, 52, and 13 different phenotypes (a phenotype is the AFLP band pattern produced by individual primer pairs) respectively in the Teshio, Shibutsu, and Tanigawa populations. The three Teshio subpopulations had a total of 17.5% polymorphic bands; the five Shibutsu subpopulations had 12.7% polymorphic bands. The AMOVA indicated that the main component of the genetic variance was among locations (87%; Table 3); we mainly observed fixed

TABLE 3. Analysis of molecular variance (AMOVA) based on 101 AFLP phenotypes from nine subpopulations at three locations. Levels of significance are based on 999 random permutations. *P*-values indicate probability of more extreme variance component estimate by chance. (SSD = sums of squares, MSD = mean squares, % = apportioned genetic variability)

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
among locations	2	1621.71	810.85	26.37	86.35	<0.001
among subpopulations	6	104.48	17.41	1.50	4.92	<0.001
within subpopulations	92	245.18	2.66	2.66	8.73	<0.001
Shibutsu						
among subpopulations	4	71.64	17.91	1.74	44.23	
within subpopulations	47	102.88	2.19	2.19	55.77	<0.001
Toikanbetsu						
among subpopulations	2	33.82	16.91	1.16	23.09	
within subpopulations	33	127.01	3.85	3.85	76.91	<0.001

bands restricted to each location. The neighbor-joining tree of the populations also showed a marked geographic structure with the Teshio and Mt. Shibutsu populations clearly separated (Fig. 3).

Clonal structure

The spatial distribution of the clonal patterns within populations are shown in Figure 4. To identify genets of *Japonolirion osense*, 48 primer combinations were used to detect variation among individuals. Three of these primer combinations were effective and yielded more polymorphic fragments than the other primer combinations. We identified genets based on these primer combinations (Table 2). In the Teshio subpopulations, each patch consisted of many genets (Fig. 4a, 4b). These clone types spread separately in disturbed, wet soil habitats. The average genet size of a plant was estimated to be 1-5 m in diameter based on the mapping data. A single clone in a small patch in Shibutsu 1, however, spread as much as 15 m (Fig. 4c). Shibutsu 1 was composed of a few genets in small patches. This habitat was genetically homogeneous compared to the habitat of other subpopulations.

Discussion

Genetic variation and population differentiation

The results of the AFLP indicated that the percentage of polymorphic fragments was very low for each location. The lower polymorphic percentage in each subpopulation was probably caused by the larger number of monotypic AFLP loci. Moreover, most of the among-location polymorphic loci were monomorphic at each location. Forty eight of the

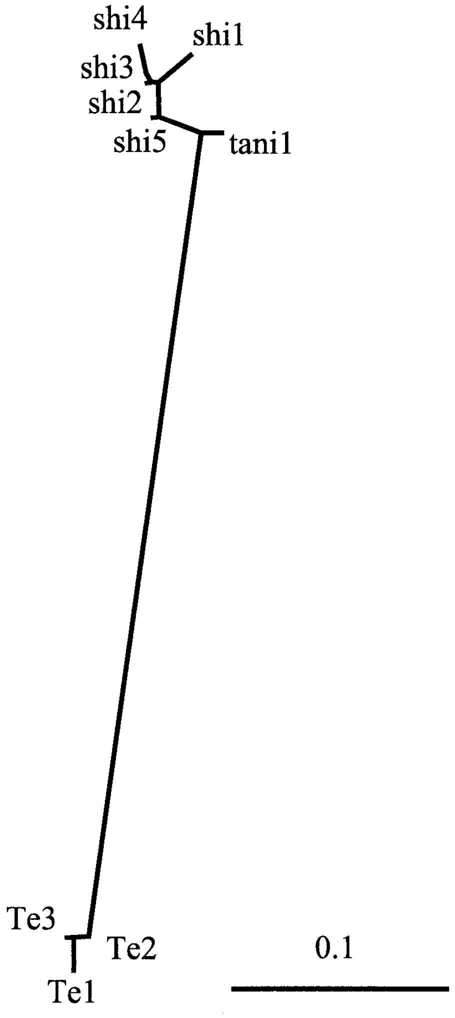

FIG. 3. Neighbor-joining tree based on Nei's (1978) genetic distance (Scale bar = 0.1 genetic distance).

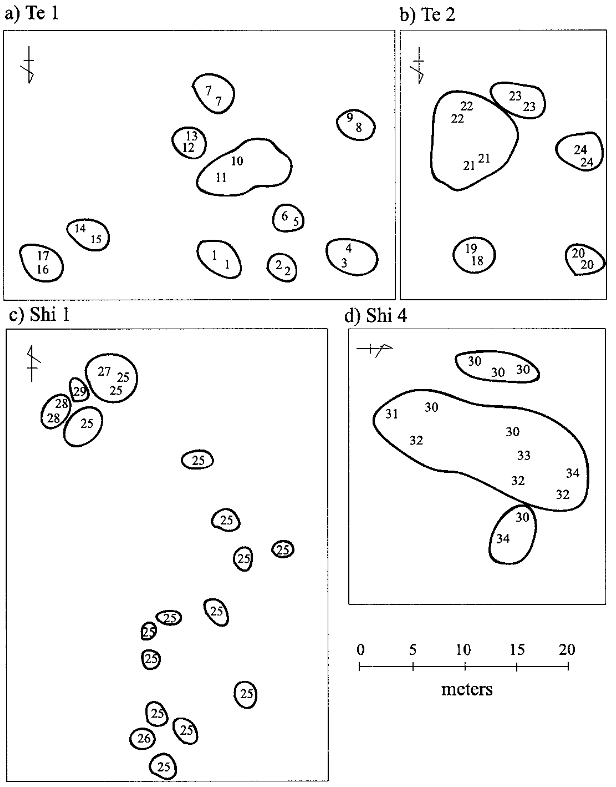

FIG. 4. Spatial distribution of phenotypes in four subpopulations of *Japonolirion osense*. Phenotypes are represented by numbers. (a) Teshio 1 and (b) Teshio 2, Toikanbetsu populations; (c) Shibutsu 1 and (d) Shibutsu 4, Mt. Shibutsu populations.

primer combinations used for primer screening showed a low degree of polymorphic bands and low variability (data not shown). Many studies (*e.g.*, Hamrick & Godt 1989) have shown that rare, geographically restricted species have lower levels of genetic diversity than widespread species, although there are several exceptions. In *Japonolirion osense* our results suggest that the disjunct habitat distribution in serpentine soils restricts plant migration and gene flow between locations, and consequently most of the genetic variation is between local populations.

We also found that plant size differed significantly between Mt. Shibutsu and Toikanbetsu. Moreover, the Neighbor-joining tree of the populations also showed that the Teshio and Mt. Shibutsu populations were widely separated. These differences in size and genetic differentiation imply that each location should be regarded as a unique genetic population and should be protected to preserve the regional distinctions.

Clonal structure within a habitat

The AFLP clonal patterns suggest that land structure effects, such as landslides caused by snow erosion, may have moved rhizomes along with soil, thereby fragmenting large habitats composed of clones into small patches in Shibutsu 1. A characteristic of this disturbed habitat is that fewer species are found in areas disturbed by landslides than in contiguous, intact habitats (data not shown), placing *Japonolirion osense* in a less-competitive situation. It has been reported that *J. osense* spreads horizontally via frequent rhizome branching (Tsukui & Takahashi 1995). If the clonal growth pattern can tolerate disturbances such as landslides, the plants can then spread by rhizomes. Clonal growth would then compensate for seedling mortality caused by disturbance, since this plant is a long-lived perennial. Clonal propagation is often a successful mechanism of population regeneration (Parker & Hamrick 1992, Mandujano *et al.* 2001). Compared to reproduction *via* rhizomes, producing new individuals in open spaces may require a substantial investment of time and cost, because no seedlings were observed between snowmelt and the end of flowering on Mt. Shibutsu. Clonal propagation, however, can increase the probability of geitonogamy and biparental inbreeding (Handel 1985, Charpentier *et al.* 2000). In particular, clonal phenotypes in the Shibutsu 1 subpopulation should be considered of a way to increase genetic diversity in terms of vegetative restoration. The open habitat on Mt. Shibutsu should be monitored further to determine the demography of *J. osense* and to evaluate the extent and frequency of disturbance.

Serpentine areas serve as biological diversity hotspots as centers for floristic differentiation and speciation (Stevanović *et al.* 2003). The genetic structure and life histories of all serpentine plants should therefore be investigated in more detail.

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